(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 February 2004 (12.02.2004)

PCT

(10) International Publication Number
WO 2004/013341 A1

(51) International Patent Classification⁷: C12P 13/08, C12N 1/21, A23K 1/16 // (C12P 13/08, C12R 1:15)

(21) International Application Number: PCT/EP2003/007474

(22) International Filing Date: 10 July 2003 (10.07.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
102 35 028.0 31 July 2002 (31.07.2002) DE

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Declaration under Rule 4.17:**

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**Published:**

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 2004/013341 A1

(54) Title: PROCESS FOR THE PRODUCTION OF L-LYSINE USING CORYNEFORM BACTERIA

(57) Abstract: The invention relates to a process for the production of L-lysine, in which the following steps are carried out: a) fermentation of the L-lysine producing coryneform bacteria that are at least resistant to diaminopimelic acid analogues, in particular 4-hydroxydiaminopimelic acid; b) enrichment of the L-lysine in the medium or in the bacterial cells; and optionally c) isolation of the L-lysine or L-lysine-containing feedstuffs additive from the fermentation broth, so that ≥ 0 to 100% of the constituents from the fermentation broth and/or from the biomass are present, and optionally bacteria are used in which in addition further genes of the biosynthesis pathway of L-lysine are enhanced, or bacteria are used in which the metabolic pathways that reduce the formation of L-lysine are at least partially switched off.

# Process for the production of L-lysine using Coryneform Bacteria

The invention provides a process for the production of L-lysine using coryneform bacteria that are resistant to diaminopimelic acid analogues, in particular 4-hydroxy-diaminopimelic acid.

Prior Art

L-amino acids, in particular L-lysine, are used in human medicine and in the pharmaceutical industry, in the foodstuffs industry and most particularly in animal nutrition.

It is known to produce amino acids by fermentation of strains of coryneform bacteria, in particular Corynebacterium glutamicum. On account of their great importance efforts are constantly being made to improve the production processes. Process improvements may relate to fermentation technology measures, such as for example stirring and provision of oxygen, or the composition of the nutrient media, such as for example the sugar concentration during the fermentation, or the working-up to the product form by for example ion exchange chromatography, or the intrinsic performance properties of the microorganism itself.

In order to improve the performance properties of these microorganisms methods involving mutagenesis, selection and choice of mutants are employed. In this way strains are obtained that are resistant to antimetabolites such as for example the lysine analogue S-(2-aminoethyl)-cysteine, or that are auxotrophic for regulatorily important metabolites and that produce L-amino acids.

For some years recombinant DNA technology methods have also been employed to improve L-amino acid producing strains of Corynebacterium glutamicum, by amplifying individual amino

acid biosynthesis genes and investigating the effect on L-amino acid production.

Object of the Invention

The inventors have been involved in devising new principles for improved processes for the fermentative production of L-lysine using coryneform bacteria.

Description of the Invention

Where L-lysine or lysine are mentioned hereinafter, this is understood to mean not only the bases, but also the salts such as for example lysine monohydrochloride or lysine sulfate.

The invention provides a process for the fermentative production of L-lysine using coryneform bacteria that are resistant to diaminopimelic acid analogues, in particular 4-hydroxydiaminopimelic acid. The analogues are generally used in concentrations of ≥ (greater than/equal to) 3 to ≤ (less than/equal to) 30 g/l.

The invention also provides a process for the fermentative production of L-lysine using coryneform bacteria that already produce L-lysine and that are resistant to diaminopimelic acid analogues, in particular 4-hydroxydiaminopimelic acid.

This invention furthermore provides a process for the production of L-lysine in which the following steps are carried out:

a) fermentation of the L-lysine producing coryneform bacteria that are at least resistant to diaminopimelic acid analogues, in particular 4-hydroxydiaminopimelic acid;

b) enrichment of the L-lysine in the medium or in the bacterial cells; and optionally

c) isolation of the L-lysine or L-lysine-containing feedstuffs additive from the fermentation broth,

so that ≥ 0 to 100% of the constituents from the fermentation broth and/or from the biomass are present.

The invention similarly provides a process for the production of coryneform bacteria that are resistant to diaminopimelic acid analogues, in particular 4-hydroxy-diaminopimelic acid.

The strains that are used produce L-lysine preferably already before the resistance to 4-hydroxydiaminopimelic acid.

The expression diaminopimelic acid analogues according to the present invention includes compounds such as

- 4-fluorodiaminopimelic acid,
- 4-hydroxydiaminopimelic acid,
- 4-oxodiaminopimelic acid, or
- 2,4,6-triaminopimelic acid.

The present invention also provides mutant coryneform bacteria producing L-lysine that are resistant to one or more of the diaminopimelic acid analogues selected from the group comprising 4-fluorodiaminopimelic acid, 4-hydroxy-diaminopimelic acid, 4-oxodiaminopimelic acid or 2,4,6-triaminopimelic acid.

The invention moreover provides feedstuffs additives based on fermentation broth that contain L-lysine produced according to the invention and no or only traces of biomass and/or constituents from the fermentation broth formed

during the fermentation of the L-lysine-producing microorganisms.

The term "traces" is understood to mean amounts of > 0% to 5%.

The invention additionally provides feedstuffs additives based on fermentation broth, characterised in that

a) they contain L-lysine produced according to the invention, and

b) they contain the biomass and/or constituents from the fermentation broth in an amount of 90% to 100% that are formed during the fermentation of the L-lysine-producing microorganisms.

The microorganisms that are provided by the present invention can produce amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. These microorganisms may be representatives of coryneform bacteria, in particular of the genus Corynebacterium. Among the genus Corynebacterium there should in particular be mentioned the species Corynebacterium glutamicum, which is known to the specialists in this field for its ability to produce L-amino acids.

Suitable strains of the genus Corynebacterium, in particular of the species Corynebacterium glutamicum, are in particular the following known wild type strains

Corynebacterium glutamicum ATCC13032
Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium melassecola ATCC17965
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067

Brevibacterium lactofermentum ATCC13869 and
Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants and/or strains produced therefrom,

such as for example the L-lysine-producing strains

Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum ATCC 21513
Corynebacterium glutamicum ATCC 21544
Corynebacterium glutamicum ATCC 21543
Corynebacterium glutamicum DSM 4697 und
Corynebacterium glutamicum DSM 5715.

It has been found that coryneform bacteria that are resistant to diaminopimelic acid analogues, in particular 4-hydroxydiaminopimelic acid, produce L-lysine in an improved manner.

In order to produce the coryneform bacteria according to the invention that are resistant to 4-hydroxydiaminopimelic acid, mutagenesis methods described in the prior art are used.

For the mutagenesis there may be employed conventional *in vivo* mutagenesis processes using mutagenic substances such as for example N-methyl-N'-nitro-N-nitrosoguanidine or ultraviolet light (Miller, J. H.: A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992).

The coryneform bacteria that are resistant to 4-hydroxy-diaminopimelic acid may be identified by plating out on

nutrient media plates containing 4-hydroxydiaminopimelic acid. End concentrations of ca. 5 to 15 g/l, for example ca. 10 g/l of 4-hydroxydiamino-pimelic acid in the nutrient medium are particularly suitable for this purpose. At this concentration mutants resistant to 4-hydroxydiaminopimelic acid may be distinguished from the unchanged parent strains by a delayed growth. After selection the mutants resistant to 4-hydroxydiaminopimelic acid exhibit an improved L-lysine production.

In addition it may be advantageous for the production of L-lysine, in addition to the resistance to 4-hydroxy-diaminopimelic acid to enhance, in particular overexpress, one or more enzymes of the respective biosynthesis pathway, glycolysis, anaplerosis, citric acid cycle, pentose phosphate cycle, amino acid export and optionally regulatory proteins. The use of endogenous genes is in general preferred.

The expressions "endogenous genes" or "endogenous nucleotide sequences" are understood to mean the genes or nucleotide sequences present in the population of a species.

The expressions "enhancement" and "to enhance" describe in this connection the increase of the intracellular activity of one or more enzymes or proteins in a microorganism that are coded by the corresponding DNA, by for example increasing the number of copies of the gene or genes, employing a strong promoter or a gene that codes for a corresponding enzyme or protein having a high activity, and optionally combining these measures.

By means of these enhancement, in particular overexpression measures, the activity or concentration of the corresponding protein is generally raised by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, at most up to 1000% or 2000%, referred to the activity or

concentration of the wild type protein and/or the activity or concentration of the protein in the starting microorganism.

Thus, for the production of L-lysine, in addition to the resistance to diaminopimelic acid analogues, in particular one or more of the genes selected from the following group may be enhanced, in particular overexpressed:

- the gene lysC coding for a feedback-resistant aspartate kinase (Accession No. P26512, EP-B-0387527; EP-A-0699759; WO 00/63388),
- the gene dapA coding for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase (Eikmanns (1992). Journal of Bacteriology 174:6076-6086),
- simultaneously the gene pyc coding for pyruvate carboxylase (DE-A-198 31 609, EP-A-1108790),
- the gene zwf coding for glucose-6-phosphate dehydrogenase (JP-A-09224661, EP-A-1108790),
- simultaneously the gene lysE coding for the lysine export protein (DE-A-195 48 222),
- the gene zwal coding for the Zwal protein (DE: 19959328.0, DSM 13115),
- the gene lysA coding for diaminopimelic acid decarboxylase (Accession No. X07563),
- the gene sigC coding for the sigma factor C (DE: 10043332.4, DSM14375),

- the gene tpi coding for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086) and

- the gene pgk coding for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086).

Furthermore it may be advantageous for the production of L-lysine, in addition to the resistance to 4-hydroxydiamino-pimelic acid, simultaneously to attenuate, in particular reduce the expression, of one or more of the genes selected from the following group:

- the gene pck coding for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),

- the gene pgi coding for glucose-6-phosphate isomerase (US 09/396,478, DSM 12969),

- the gene poxB coding for pyruvate oxidase (DE:1995 1975.7, DSM 13114),

- the gene deaD coding for DNA helicase (DE: 10047865.4, DSM14464),

- the gene citE coding for citrate lysase (PCT/EP01/00797, DSM13981),

- the gene menE coding for O-succinylbenzoic acid CoA-ligase (DE: 10046624.9, DSM14080),

- the gene mikE17 coding for the transcription regulator MikE17 (DE: 10047867.0, DSM14143) and

- the gene zwa2 coding for the Zwa2 protein (DE: 19959327.2, DSM 13113).

The term "attenuation" describes in this connection the reduction or switching off of the intracellular activity of one or more enzymes (proteins) in a microorganism that are

coded by the corresponding DNA, by using for example a weak promoter or a gene or allele that codes for a corresponding enzyme with a low activity or inactivating the corresponding gene or enzyme (protein), and optionally combining these measures.

By means of these attenuation measures the activity or concentration of the corresponding protein is generally reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild type protein, and/or the activity or concentration of the protein in the initial microorganism.

Finally it may be advantageous for the production of L-lysine, in addition to the resistance to 4-hydroxy-diaminopimelic acid, also to switch off undesirable secondary reactions (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The microorganisms produced according to the invention are also covered by the invention and may be cultivated continuously or discontinuously in a batch process (batch cultivation) or in a fed-batch process (feed process) or repeated fed-batch process (repetitive feed process) for the purposes of producing L-lysine. A summary of known cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Brunswick/ Wiesbaden, 1994)).

The culture medium to be used must satisfy in a suitable manner the requirements of the respective strains. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for

General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

As carbon source there may be used sugars and carbohydrates such as for example glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats such as for example soy bean oil, sunflower oil, groundnut oil and coconut oil, fatty acids such as for example palmitic acid, stearic acid and linoleic acid, alcohols such as for example glycerol and ethanol, and organic acids such as for example acetic acid. These substances may be used individually or as a mixture.

As nitrogen source there may be used organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soy bean flour and urea, or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or as a mixture.

As phosphorus source there may be used phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts. The culture medium must furthermore contain salts of metals, such as for example magnesium sulfate or iron sulfate, that are necessary for growth. Finally, essential growth promoters such as amino acids and vitamins may be used in addition to the aforementioned substances. Apart from these, suitable precursors may be added to the culture medium. The aforementioned starting substances may be added to the culture in the form of a single batch or may be fed in in an appropriate manner during the cultivation.

In order to regulate the pH of the culture basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water, or acidic compounds such as phosphoric acid or sulfuric acid are used as appropriate. In order to

control foam formation antifoaming agents such as for example fatty acid polyglycol esters may be used. In order to maintain the stability of plasmids, suitable selectively acting substances, for example antibiotics, may be added to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures such as for example air are fed into the culture. The temperature of the culture is normally 20°C to 45°C, and preferably 25°C to 40°C. Cultivation is continued until a maximum amount of desired product has been formed. This target is normally achieved within 10 hours to 160 hours.

Methods for the determination of L-lysine are known from the prior art. The analysis may be carried out as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography followed by ninhydrin derivatisation, or by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention serves for the fermentative production of L-lysine.

The concentration of L-lysine may optionally be adjusted to the desired value by the addition of L-lysine.

By means of the described processes it is possible to isolate coryneform bacteria that are resistant to diaminopimelic acid analogues, in particular 4-hydroxy-diaminopimelic acid, and to produce L-lysine in an improved manner according to the described fermentation processes.

Example 1

Screening for clones resistant to 4-hydroxydiaminopimelic acid.

The Corynebacterium glutamicum strain DM1725 was produced by multiple untargeted and targeted mutagenesis including genetic engineering methods, selection and mutant selection from C. glutamicum ATCC13032. The strain is resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and has two identical complete copies of the LysC gene that code for a feedback-resistant aspartate kinase. The two copies are located at the LysC gene site on the chromosome. The feedback-resistant aspartate kinase is insensitive to inhibition by mixtures of lysine (or the lysine analogue S-(2-aminoethyl)-L-cysteine, 100mM) and threonine (10mM), but in contrast to this the activity of aspartate kinase in the wild type is inhibited up to 10% residual activity. The strain is streptomycin resistant.

A pure culture of the strain DM1725 was deposited as DSM 15662 on 6 June 2003 at the German Collection for Microorganisms and Cell Cultures (DSM Brunswick) according to the Budapest Convention.

For screening on colonies that are resistant to 4-hydroxy-diaminopimelic acid, the strain DSM 15662 after UV mutagenesis (Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd Edition, Cold Spring Harbor, New York, 1989) is plated out on LB agar plates containing 4-hydroxydiaminopimelic acid. The agar plates are supplemented with 10 g/l of 4-hydroxydiaminopimelic acid. The growth of the colonies is observed over 48 hours. At this concentration mutants that are resistant to 4-hydroxy-diaminopimelic acid can be distinguished from the unaltered parent strain by an improved growth. In this way a clone is identified that exhibits a much better growth compared

to DSM 15662. The strain is identified as DSM 15662_Hdap_r.

Example 2

Production of lysine

The C. glutamicum strain DSM 15662_Hdap_r obtained in Example 1 is cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant is determined.

For this purpose the strains are first of all incubated on agar plates for 24 hours at 33°C. Using this agar plate culture a preculture is inoculated (10 ml of medium in a 100 ml Erlenmeyer flask). The medium MM is used as medium for the preculture. The preculture is incubated for 24 hours at 33°C at 240 rpm on a vibrator. Using this preculture a main culture is inoculated so that the initial optical density (OD - 660 nm) of the main culture is 0.1 OD. The medium MM is also used for the main culture.

Medium MM

| | |
|---|---|
| CSL | 5 g/l |
| MOPS | 20 g/l |
| Glucose (separately autoclaved) | 50 g/l |
| Salts: | |
| $(NH_4)_2SO_4$ | 25 g/l |
| $KH_2PO_4$ | 0.1 g/l |
| $MgSO_4$ x 7 $H_2O$ | 1.0 g/l |
| $CaCl_2$ x 2 $H_2O$ | 10 mg/l |
| $FeSO_4$ x 7 $H_2O$ | 10 mg/l |
| $MnSO_4$ x $H_2O$ | 5.0 mg/l |
| Biotin (sterile filtered) | 0.3 mg/l |
| Thiamine x HCl (sterile filtered) | 0.2 mg/l |
| $CaCO_3$ | 25g/l |

CSL (Corn Steep Liquor), MOPS (morpholinopropanesulfonic acid) and the salt solution are adjusted with ammonia water to pH 7 and autoclaved. The sterile substrate and vitamin solutions as well as the dry autoclaved $CaCO_3$ are then added.

Culturing is carried out in a 10 ml volume in a 100 ml Erlenmeyer flask equipped with baffles. The culturing is carried out at 33°C and 80% atmospheric humidity.

After 72 hours the OD is determined at a measurement wavelength of 660 nm with a Biomek 1000 instrument (Beckmann Instruments GmbH, Munich). The amount of lysine formed is determined by ion exchange chromatography and

post-column derivatisation with ninhydrin detection, using an amino acid analyser from Eppendorf-BioTronik (Hamburg, Germany).

The result of the experiment is shown in Table 1

Table 1

| Strain | OD (660 nm) | Lysine. HCl g/l |
|---|---|---|
| DSM 15662 | 11.6 | 16.2 |
| DSM 15662_Hdap_r | 11.9 | 18.9 |

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Degussa AG
Kantstr. 2
33790 Halle/Westf.

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

| I. IDENTIFICATION OF THE MICROORGANISM | |
|---|---|
| Identification reference given by the DEPOSITOR: DM1725 | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 15662 |
| II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION | |
| The microorganism identified under I. above was accompanied by:<br>( x ) a scientific description<br>( x ) a proposed taxonomic designation<br>(Mark with a cross where applicable). | |
| III. RECEIPT AND ACCEPTANCE | |
| This International Depositary Authority accepts the microorganism identified under I. above, which was received by it on 2003-06-06 (Date of the original deposit)[1]. | |
| IV. RECEIPT OF REQUEST FOR CONVERSION | |
| The microorganism identified under I above was received by this International Depositary Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion). | |
| V. INTERNATIONAL DEPOSITARY AUTHORITY | |
| Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH<br>Address: Mascheroder Weg 1b<br>D-38124 Braunschweig | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):<br>V. Weiss<br>Date: 2003-06-10 |

[1] Where Rule 6.4 (d) applies, such date is the date on which the status of international depositary authority was acquired.

Form DSMZ-BP/4 (sole page) 12/2001

**BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE**

**INTERNATIONAL FORM**

Degussa AG
Kantstr. 2
33790 Halle/Westf.

**VIABILITY STATEMENT**
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

| I. DEPOSITOR | II. IDENTIFICATION OF THE MICROORGANISM |
|---|---|
| Name: Degussa AG<br>Kantstr. 2<br>Address: 33790 Halle/Westf. | Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:<br>DSM 15662<br>Date of the deposit or the transfer[1]:<br>2003-06-06 |

| III. VIABILITY STATEMENT |
|---|
| The viability of the microorganism identified under II above was tested on 2003-06-06<br>On that date, the said microorganism was<br>(x)[3] viable<br>( )[3] no longer viable |

| IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4] |
|---|
| |

| V. INTERNATIONAL DEPOSITARY AUTHORITY | |
|---|---|
| Name: DSMZ-DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH<br>Address: Mascheroder Weg 1b<br>D-38124 Braunschweig | Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):<br>V. Weiß<br>Date: 2003-06-10 |

1 Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2 In the cases referred to in Rule 10.2(a) (ii) and (iii), refer to the most recent viability test.
3 Mark with a cross the applicable box.
4 Fill in if the information has been requested and if the results of the test were negative.

Form DSMZ-BP/9 (sole page) 12/2001

**Patent Claims**

1. Process for the production of L-lysine, characterised in that the following steps are carried out:

a) fermentation of the L-lysine producing coryneform bacteria that are at least resistant to diaminopimelic acid analogues, in particular 4-hydroxydiaminopimelic acid;

b) enrichment of the L-lysine in the medium or in the bacterial cells; and optionally

c) isolation of the L-lysine or L-lysine-containing feedstuffs additive from the fermentation broth,

so that ≥ 0 to 100% of the constituents from the fermentation broth and/or from the biomass are present.

2. Process according to claim 1, characterised in that bacteria are used in which in addition further genes of the biosynthesis pathway of L-lysine are enhanced.

3. Process according to claim 1, characterised in that bacteria are used in which the metabolic pathways that reduce the formation of L-lysine are at least partially switched off.

4. Process according to claim 1, characterised in that for the production of L-lysine coryneform microorganisms are fermented in which at the same time one or more of the genes selected from the following group is/are enhanced, in particular overexpressed:

4.1 the gene lysC coding for a feedback-resistant aspartate kinase,

4.2 the gene dapA coding for dihydrodipicolinate synthase,

4.3 the gene gap coding for glyceraldehyde-3-phosphate dehydrogenase,

4.4 the gene pyc coding for pyruvate carboxylase,

4.5 the gene zwf coding for glucose-6-phosphate dehydrogenase,

4.6 simultaneously the gene lysE coding for the lysine export protein,

4.7 the gene zwa1 coding for the Zwa1 protein,

4.8 the gene lysA coding for diaminopimelic acid decarboxylase,

4.9 the gene sigC coding for the sigma factor C,

4.10 the gene tpi coding for triose phosphate isomerase, or

4.11 the gene pgk coding for 3-phosphoglycerate kinase.

5. Process according to claim 1, characterised in that for the production of L-lysine coryneform microorganisms are fermented in which at the same time one or more of the genes selected from the following group is/are attenuated:

5.1 the pck gene coding for phosphoenol pyruvate carboxykinase,

5.2 the pgi gene coding for glucose-6-phosphate-isomerase,

5.3 the gene deaD coding for DNA helicase,

5.4 the gene citE coding for citrate lysase,

5.5 the gene menE coding for O-succinylbenzoic acid CoA-ligase,

5.6 the gene mikE17 coding for the transcription regulator MikE17,

5.7 the gene poxB coding for pyruvate oxidase, or

5.8 the gene zwa2 coding for the Zwa2 protein.

6. Process according to one or more of the preceding claims, characterised in that microorganisms of the species Corynebacterium glutamicum are used.

7. Process according to one or more of the preceding claims, characterised in that microorganisms of the species Corynebacterium glutamicum that are resistant to 4-hydroxydiaminopimelic acid are used.

8. Mutants of coryneform bacteria producing L-lysine and that are resistant to one or more of the diamino-pimelic acid analogues selected from the group comprising 4-fluorodiamino-pimelic acid, 4-hydroxydiaminopimelic acid, 4-oxo-diaminopimelic acid or 2,4,6-triaminopimelic acid.

9. Process according to claims 1 to 7, characterised in that mutants of coryneform bacteria are used that produce L-lysine and that are resistant to one or more of the diaminopimelic acid analogues selected from the group comprising 4-fluorodiaminopimelic acid, 4-hydroxydiaminopimelic acid, 4-oxo-diaminopimelic acid or 2,4,6-triaminopimelic acid.

10. Feedstuffs additives based on fermentation broth, characterised in that

a) they contain L-lysine produced according to claims 1 to 7 or 9, and

b) they contain the biomass and/or constituents from the fermentation broth formed during the fermentation of the L-lysine-producing microorganisms in an amount of 0% to 5%.

11. Feedstuffs additives based on fermentation broth, characterised in that

a) they contain L-lysine produced according to claims 1 to 7 or 9, and

they contain the biomass and/or constituents from the fermentation broth formed during the fermentation of the L-lysine-producing microorganisms in an amount of 90% to 100%.

# INTERNATIONAL SEARCH REPORT

PCT/EP 03/07474

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7 C12P13/08 C12N1/21 A23K1/16 //(C12P13/08,C12R1:15)

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P C12N A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, FSTA, CHEM ABS Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 24 November 2003 | 04/12/2003 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Devijver, K |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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